BRAIN COMMUNICATIONS

Genome sequencing identifies rare tandem repeat expansions and copy number variants in Lennox–Gastaut syndrome

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Epilepsies are a group of common neurological disorders with a substantial genetic basis. Despite this, the molecular diagnosis of epilepsies remains challenging due to its heterogeneity. Studies utilizing whole-genome sequencing may provide additional insights into genetic causes of epilepsies of unknown aetiology. Whole-genome sequencing was used to evaluate a cohort of adults with unexplained developmental and epileptic encephalopathies ($n = 30$), for whom prior genetic tests, including whole-exome sequencing in some cases, were negative or inconclusive. Rare single nucleotide variants, insertions/deletions, copy number variants and tandem repeat expansions were analysed. Seven pathogenic or likely pathogenic single nucleotide variants, and two pathogenic deleterious copy number variants were identified in nine patients (32.1% of the cohort). One of the copy number variants, identified in a patient with Lennox–Gastaut syndrome, was too small to be detected by chromosomal microarray techniques. We also identified two tandem repeat expansions with clinical implications in two other patients with Lennox–Gastaut syndrome: a CGG repeat expansion in the 5′untranslated region of *DIP2B,* and a CTG expansion in *ATXN8OS* (previously implicated in spinocerebellar ataxia type 8). Three patients had *KCNA2* pathogenic variants. One of them died of sudden unexpected death in epilepsy. The other two patients had, in addition to a *KCNA2* variant, a second *de novo* variant impacting potential epilepsy-relevant genes (*KCNIP4* and *UBR5*). Overall, whole-genome sequencing provided a genetic explanation in 32.1% of the total cohort. This is also the first report of coding and non-coding tandem repeat expansions identified in patients with Lennox–Gastaut syndrome. This study demonstrates that using whole-genome sequencing, the examination of multiple types of rare genetic variation, including those found in the non-coding region of the genome, can help resolve unexplained epilepsies.

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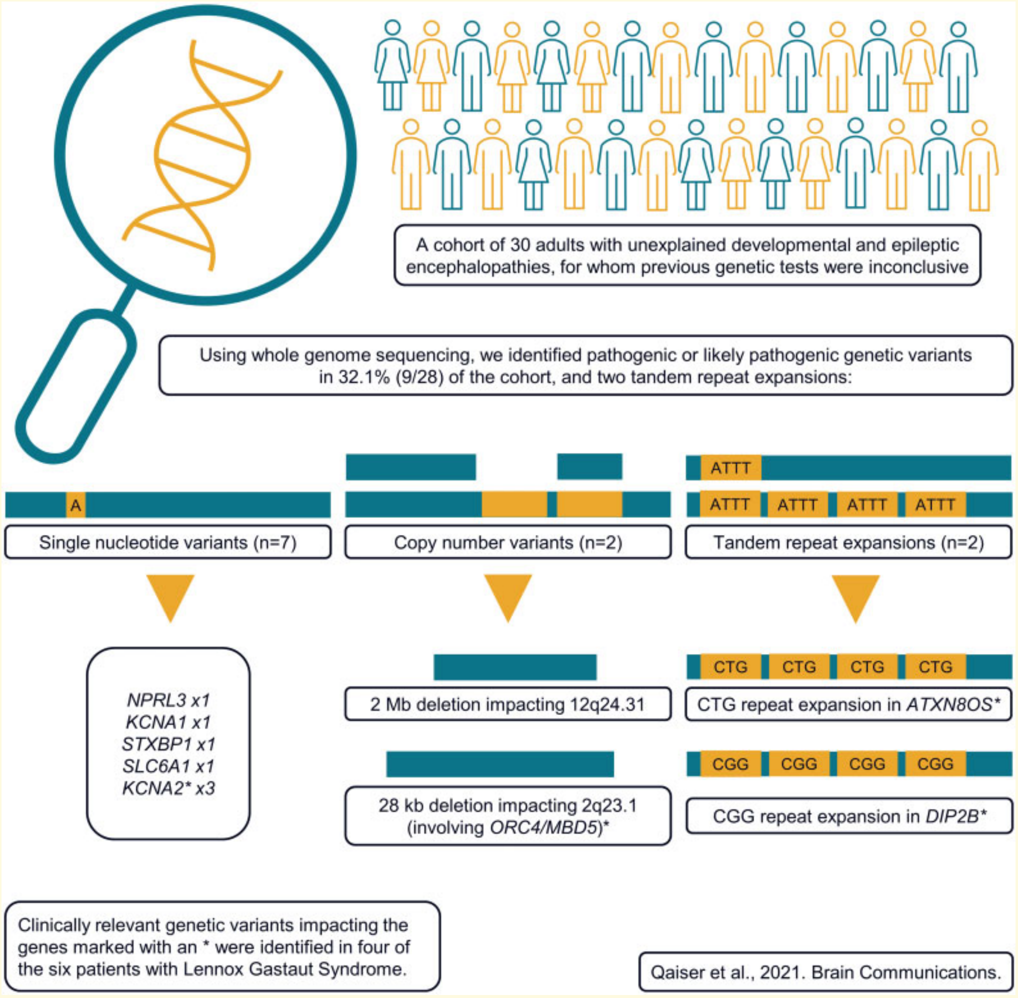
Abbreviations: ACMG =American College of Medical Genetics and Genomics; ASD =autism spectrum disorder; BAFME =benign familial adult myoclonic epilepsy; bp =base pair; c. =coding DNA change; Chr =chromosome; CMA =chromosomal microarray; CNV =copy number variant; DD =developmental delay; DEE =developmental epileptic encephalopathy; DR =developmental regression; GATK =Genome Analysis Toolkit; GQ =genotype quality; GoF =gain-of-function; ID =intellectual disability; LGS =Lennox–Gastaut Syndrome; LoF =loss-of-function; MAND =*MBD5*-associated neurodevelopmental disorder; NDD =neurodevelopmental disorder; OR =odds-ratio; p =protein change; PC =principal component; RP-PCR =repeat-primed PCR; SCA =spinocerebellar ataxia; SNP =single nucleotide polymorphism; SNV =single nucleotide variant; SUDEP =sudden unexpected death in epilepsy; TR =tandem repeat; TRF =Tandem Repeat Finder; UTR =untranslated region; VUS =variant of unknown significance; WGS =whole-genome sequencing; WES =whole-exome sequencing.

Graphical Abstract



A cohort of 30 adults with unexplained developmental and epileptic encephalopathies, for whom previous genetic tests were inconclusive

Using whole genome sequencing, we identified pathogenic or likely pathogenic genetic variants in 32.1% (9/28) of the cohort, and two tandem repeat expansions:

A
Single nucleotide variants (n=7)
NPRL3 x1
KCNA1 x1
STXBP1 x1
SLC6A1 x1
KCNA2 x3*
Copy number variants (n=2)
2 Mb deletion impacting 12q24.31
28 kb deletion impacting 2q23.1 (involving *ORC4/MBD5*)*
ATTT
ATTT ATTT ATTT ATTT
Tandem repeat expansions (n=2)
CTG CTG CTG CTG
CTG repeat expansion in *ATXN8OS**
CGG CGG CGG CGG
CGG repeat expansion in *DIP2B**

Clinically relevant genetic variants impacting the genes marked with an * were identified in four of the six patients with Lennox Gastaut Syndrome.

Qaiser et al., 2021. Brain Communications.

Introduction

Epilepsies are a group of common neurological disorders which are primarily characterized by their enduring predisposition to generate recurrent seizures.[1–3] Genetic factors contribute to the aetiology of epilepsy, where to date, over 900 epilepsy-associated genes have been identified.[3–7] Interestingly, only a small proportion of known epilepsy-associated genes encode for ligand (10%) and voltage-gated (17%) ion channels.[4–7] New genetic mechanisms continue to be implicated in both focal and generalized epilepsies.[3,8–12] For example, Lennox–Gastaut Syndrome (LGS) is a severe childhood-onset epilepsy syndrome that may be caused by acquired mechanisms (e.g. brain injuries and hypoxic ischaemic lesions), structural abnormalities and/or genetic causes.[13,14]

Despite this known genetic basis, the molecular diagnosis of epilepsies remains challenging, with current studies capturing only a portion of the contributing genetic variation.[5,15] This can partially be attributed to the incomplete assessment of genetic variants by most current genetic analysis methods. For example, in addition to single nucleotide variation, intronic $(TTTTA)_n$/$(TTTCA)_n$ tandem repeat (TR) expansions have recently been implicated in benign familial adult myoclonic epilepsy (BAFME), suggesting that TR expansions may be a more common mechanism in epilepsies than currently reported.[16–19] Studies interrogating the full spectrum of genomic variants, especially those utilizing whole-genome sequencing (WGS), will provide insights into epilepsy genetics and help resolve epilepsies of unknown aetiology.

In this study, we used WGS to identify rare genetic variation in a cohort of adults with developmental epileptic encephalopathies (DEEs), for whom all prior genetic tests were negative or inconclusive.

Materials and methods

Cohort recruitment

Adults with DEEs ($n = 30$) were recruited from the Adult Epilepsy Genetics Program at Toronto Western Hospital. All patients had a prior negative or inconclusive genetic test. This study was approved by the University Health Network's Research Ethics Board.

Whole-genome sequencing

Of the 30 patients, there were two pairs of identical twins. Parents were also sequenced where possible ($n = 11$ pairs). In brief, genomic DNA was extracted from whole blood, and then sequenced using the Illumina HiSeq X platform at The Centre for Applied Genomics. Sequence reads were aligned to the GRCh37 reference genome using the Burrows-Wheeler Aligner read alignment program.[20] Local realignment, base quality recalibration and removal of duplicate reads were carried out using the Broad Institute's Genome Analysis Toolkit (GATK) package. The following algorithms were used for variant calling and genotyping: (i) GATK Haplotype Caller for single nucleotide variants (SNVs) and indels; (ii) ERDS (v1.1) and CNVnator (v0.3.2) for copy number variants (CNVs); and (iii) a combination of ExpansionHunter Denovo (EHdn, v0.7), Tandem Repeat Finder (TRF) and ExpansionHunter (EH, v3.0.2) for TRs.[21–23]

Each variant call format file was annotated using a custom ANNOVAR pipeline.[24] To filter for only high quality SNVs, the following parameters were applied: (i) autosomal heterozygous variants met a genotype quality (GQ) cut-off value of ≥ 99, and an alternative allele fraction ≥ 0.3 and ≤ 0.7; (ii) homozygous or chromosome X variants had a GQ $\geq$ 25 and an alternative allele fraction >0.7; and (iii) all variants passed GATK pipeline filter constraints. DeNovoGear was used to detect *de novo* SNVs in patients sequenced in trio ($n = 13$, where two of the families have twins).[25] To filter for only robust CNVs, CNVs had to be called by both ERDS and CNVnator.[21] For TRs, EHdn was used to estimate the size and location of TRs lengths in >60 known TR-associated disease loci, as previously described.[23,26] Ancestry and kinship analyses were carried out using PLINK (v1.9), as previously described.[27]

Rare variant analysis

A rare variant was defined as being present with <1% frequency in the general population, using genomic databases (the 1000 Genomes project, Exome Aggregation Consortium, Genome Aggregation Database, DECIPHER and the Database of Genomic Variants). Rare SNVs and CNVs impacting both coding and non-coding regions of the genome were analysed, as previously described.[27,28] TR expansions were filtered based on the genomic content disrupted, biological relevance, known disease associations, and any overlap with reported literature or clinical reports in databases. Using the American College of Medical Genetics and Genomics' (ACMG) guidelines, we classified rare SNVs and CNVs into pathogenic (i.e. disease-causing), likely pathogenic, variant of unknown significance (VUS), benign and likely benign categories.[29–32] If a VUS was identified in a gene without a validated association to the patient's phenotype, it was considered a gene of uncertain significance.[29] As there are no ACMG standards to guide the interpretation of the pathogenicity of TR expansions, we defined a repeat expansion to be of interest if its size either (i) fell into the reported disease-causing range, or (ii) was larger than what is observed in control individuals in reported studies.

Variant validation

Where possible, variants were experimentally validated using either (i) Sanger Sequencing for SNVs; (ii) qPCR for CNVs; or (iii) Southern blotting and/or repeat-primed

PCR (RP-PCR) for TR expansions. Specifically, southern blotting was used to validate the CGG expansion in *DIP2B* found in patient 4 as previously described.[33] In brief, genomic DNA was first digested using selected restriction-endonuclease enzymes, followed by the resolution of DNA fragments on an agarose gel. These DNA fragments were then transferred to a nylon membrane, and then subjected to hybridization analysis, where radioactively labelled probes were used to detect the sizes of the CGG alleles in *DIP2B*.

To validate the CTG repeat expansion in *ATXN80S* in both patient 6 and his mother (6A), a combination of primer sequences were used to amplify the CTG repeat locus in *ATXNN8OS*: a single forward fluorescently labelled primer (P1, 5′-6-FAM—CTGGGTCCTTCATGTT AGAAAACCT-3′), and a combination of two reverse primers in a 1:10 ratio (P3, 5′-TACGCATCCCAGTTT GAGACGC-3′, and P4, 5′-TACGCATCCCAGTTTG AGACGCAGCAGCAGCAGCAGCAGCA-3′).[34,35] The reverse primer P4 anneals at different sites along the CTG repeat, resulting in a range of PCR product sizes up to the inclusion of the full repeat, depending on the amplification limit of the assay. These fluorescently labelled PCR products of different lengths were analysed using capillary electrophoresis and visualized using PeakScanner 2.

Statistical analysis: comparison of clinical characteristics

One-sided 2×2 Fisher Exact Tests were carried out to compare clinical characteristics between patients with an identified variant, and those with no identified variant, including sex, age of seizure onset, EEG, MRI, presence/absence of family history, developmental regression (DR) and intellectual disability (ID) severity. In each case, an odds-ratio (OR) and a *P*-value (*P*) were generated.

Data availability

The dataset(s) supporting the conclusions of this article is(are) included within the manuscript. Individual ancestries and kinship values are not included in order to preserve participants' privacy.

Results

The cohort had 30 patients in total (19 males), all with DEEs. Six patients were diagnosed with LGS. The median age of seizure onset was 3 years (range: 17 days to 17 years old). Common clinical characteristics included epileptiform interictal findings in their paediatric or adult EEGs ($n = 24$, 80%), ID ($n = 30$, 100%), autism spectrum disorder (ASD, $n = 10$, 33.3%), and a previous family history of epilepsy or a neurodevelopmental disorder (NDD) ($n = 19$, 63.3%). Seven patients had psychiatric comorbidities, such as ADHD, anxiety and schizophrenia ($n = 7$, 23.3%). This cohort includes one adopted patient (Patient 19), one offspring of a consanguineous marriage (Patient 16) and two pairs of identical twins (Patients 8 & 12; Patients 29 & 30). All patients had previously undergone extensive investigation during their paediatric years. During their years in the Toronto Western Hospital's Adult Epilepsy Genetics Program, the patients completed chromosomal microarrays (CMAs) ($n = 25$, 83.3%), large gene panels ($n = 25$, 83.3%) or whole-exome sequencing (WES) ($n = 5$, 16.7%).

WGS analysis

In total, 52 genomes were sequenced: 30 patients and 22 parents (Supplementary Table 1, Supplementary Fig. 1). There was an average read depth of 37.0 across the entire cohort. Overall, WGS detected an average of 4 535 791 indels and SNVs, and 706 CNVs per genome amongst patients. Regarding rare variants, patients had an average of 307 indels and SNVs, and 4.1 CNVs impacting coding regions per genome.

Ancestry analyses found that the cohort was largely European ($n = 40$, 76.9%), with the remainder of patients belonging to South Asian ($n = 9$, 17.3%), American ($n = 2$, 3.85%) and East Asian ($n = 1$, 1.92%) ancestry groups (Supplementary Fig. 2). We confirmed that patient 16's parents (16A, 16B) were third-degree relatives, and that the two twin pairs were genetically identical. Only one individual in each twin pair was included in downstream analyses, resulting in a total of 28 patients' WGS data for analysis.

Clinically relevant rare variant analysis

Upon rare variant analysis, we identified seven pathogenic or likely pathogenic SNVs, and two pathogenic CNVs, resulting in a molecular diagnosis in 9/28 (32.1%) patients (Tables 1 and 2).

In addition, we identified two *de novo* VUS SNVs, which do not meet clinical significance as per ACMG guidelines, but instead are potentially clinically relevant and merit further investigation. We also identified two TR expansions in *DIP2B and ATXN8OS*. Overall, patients with an identified variant had a younger age of seizure onset compared to the variant absent group (OR: 8.27, $P = 0.042$, one-sided Fisher's Exact Test) (Table 3).

Single nucleotide variants

Patient 24: We identified a novel rare pathogenic variant (p.Gln96fs) in *NPRL3*, of unknown inheritance, which results in a frameshift deletion. Heterozygous loss-of-function (LoF) variants in *NPRL3* have been implicated in autosomal dominant focal epilepsies.[12]

Table 1 Clinical descriptions of patients with identified genetic variants

Patient	Sex	Syndrome	Age of seizure onset (years)	Phenotype description
24	M	DEE	17	A 34-year-old patient has epilepsy, with abnormal EEG (unclear if frontal or generalized), mild ID and ASD diagnosis. Patient's mother, maternal aunt and uncle had seizures but were not available for testing.
16	F	Early onset epileptic encephalopathy	0.5 (6 months)	A 18-year-old patient has multifocal epilepsy, abnormal EEG, developmental delay (motor, speech, and language), severe ID and ASD diagnosis. Patient's brother presented with seizures in early childhood but is seizure free. Mother has mild ID.
29; 30*	M	Early onset epileptic encephalopathy	0.12 (6 weeks)	34-year-old identical twins with an epilepsy of unclear localization, who presented with an abnormal EEG and normal MRI, developmental delay (motor, speech, and language), severe ID, dysmorphism and severe spasticity. Both were born prematurely. Patient 30 is blind. Patient 29 presented with DR.
9	M	Early onset epileptic encephalopathy	1.5	A 30-year-old patient has myoclonic-atonic seizures, with an abnormal EEG, severe ID and severe ASD diagnosis (at age three years old). There is no family history of epilepsy. The patient's sister also has an ASD diagnosis.
19	F	Early onset epileptic encephalopathy	0.67 (8 months)	Patient had a multifocal epilepsy, with an abnormal EEG and normal MRI, mild ID, depression and a mild ASD diagnosis. Patient passed away from SUDEP at 26 years old. Family history is unknown as the patient was adopted.
3	F	Early onset epileptic encephalopathy	1	A 40-year-old patient has an epilepsy with seizure onset not localizable, with an abnormal EEG, severe ID, speech delay, dystonia and ataxia. Patient's father's first cousin had developmental delay and seizures.
27	F	LGS	0.05 (17 days)	A 17-year-old patient has a multifocal epilepsy, slow generalized spike and wave, generalized paroxysmal fast activity and MISF. MRI is normal; Developmental delay (motor, speech and language), hypotonia, DR, dysmetria and dysdiadokokinesia, severe ID and an ASD diagnosis. There is no family history of epilepsy. Patient's brother has ADHD.
10	F	LGS	3	A 23-year-old patient has multifocal and generalized seizures. EEGs show generalized paroxysmal fast activity, slow generalized spike, and wave discharges, MISF and generalized slow background activity. Patient has developmental delay (speech and language), later also had DR, moderate ID, short stature, weight and high below the 3rd percentile, and a movement disorder. She was diagnosed with diffuse muscle weakness and truncal instability, collapsing kyphosis, hypogonadism, and growth hormone deficiency. She was conceived through in vitro fertilization. Her twin sister died in uterus due to alobar anencephaly. This patient was a hypotonic baby. She sat at one-year-old, walked at two years, said her first words at four years, and was toilet trained at six years. The patient had moderate ID, and at the age of eight years, she started showing regression. She is completely non-verbal, inconsistently makes eye contact, is wheelchair bound, has lost sphincter control, and is fed through a G-tube.
26	F	DEE	3	A 17-year-old patient has multifocal epilepsy with abnormal EEG and normal MRI, developmental delay (speech and language), and severe ID. Patient's brother had two episodes of seizures at the age of nine years old.
6	M	LGS	4	A 26-year-old patient has LGS with multifocal and generalized epilepsy, EEG showing slow generalized spike and wave, generalized paroxysmal fast activity and MISF and MRI is normal, mild ID, and a ASD diagnosis. There is no family history of epilepsy or ataxia.

(continued)

Table 1 Continued

Patient	Sex	Syndrome	Age of seizure onset (years)	Phenotype description
4	M	LGS	1	Born at term after an uncomplicated pregnancy. A 20-year-old patient has LGS with multifocal and generalized epilepsy, EEG showing slow generalized spike and wave, generalized paroxysmal fast activity, MISF and slow background. Normal MRI, developmental delay (speech and language), later also had DR and moderate ID. Patient's father has nocturnal seizures and has been treated with carbamazepine since the age of 14 years old.

The * indicates that patients 29 and 30 are identical (i.e. monozygotic) twins. DR, developmental regression; LGS: Lennox–Gastaut syndrome; MISF, multiple independent spike foci; SUDEP, sudden unexpected death in epilepsy.

Table 2 Molecular findings identified by WGS in this cohort

Patient	Variant type	Gene	Variant	Coordinate; Transcript	De Novo or Inherited	Interpretation; Evidence
24	SNV	*NPRL3*	p.Gln96fs; c.287delA	Chr16:162,646; NM_001243247	NA	Pathogenic: PVS1, PM2, PP4
16	SNV	*KCNA1*	p.Gln426Ter; c.C1276T	Chr12:5,021,820; NM_000217	Maternally inherited	Likely Pathogenic: PVS1_ Moderate, PM2
29; 30*	SNV	*STXBP1*	p.Arg292His; c.G875A	Chr9:130,430,439; NM_003165	*De novo*	Likely Pathogenic: PS2, PM2, PP2, PP3, PP4
9	SNV	*SLC6A1*	c.Gly889Cys; p. G297R	Chr3:11,067,498; NM_003042	NA	Pathogenic: PS1, PM2, PM5, PP2, PP3, PP4
19	SNV	*KCNA2*	p.Lys445fs; c.1334delA	Chr1:111,146,070; NM_004974	NA	Pathogenic: PVS1, PM2, PP4
3	SNV	*KCNA2*	p.Arg297Gln; c.G890A	Chr1:111,146,515; NM_001204269	*De novo*	Pathogenic: PS1, PS2, PM2, PP2, PP3, PP4
	SNV	*KCNIP4*	p.Gln96Ter; c.C286T	Chr4:20,736,316; NM_001035004	*De novo*	VUS: PS2, PM2
27	SNV	*KCNA2*	p.Glu183Lys; c.G547A	Chr1:111,146,858; NM_004974	*De novo*	Likely Pathogenic: PS2, PM2, PP2, PP3, PP4
	SNV	*UBR5*	p.Pro1309Ser; c.C3925T	Chr8:103,307,648; NM_001282873	*De novo*	VUS: PS2, PM2
10	CNV	*ORC4* | *MBD5*	DEL	Chr2:148,759,580 −148,787,852 Size: 28,273 bp	NA	Pathogenic: 1A, 2C-1, 3A, 4B/C, 5F
26	CNV	38 genes	DEL	Chr12:120,867,801–123,012,800 Size: 2,145,000	NA	Likely Pathogenic: 1A, 3A, 4C, 5F
6	TR	*ATXN8OS*	19 and ∼135 CTG units	Chr13: 70713516 −70713561	NA	NA
4	TR	*DIP2B*	12 and 128 CGG units	Chr12:50898787 −50898807	NA	NA

All variants are described in relation to the coding DNA reference sequence (i.e. 'c.'), the predicted consequences on the protein level ('p.'), and transcript ('NM_'). Variant interpretation is according to the ACMG guidelines (see Methods).

The * indicates that patients 29 and 30 are identical (i.e. monozygotic) twins. bp, base pair; Chr, chromosome; CNV, copy number variant; del/DEL, deletion; c, coding DNA; NA, not applicable; p, protein; SNV, single nucleotide variant; TR, tandem repeat; VUS, variant of uncertain significance.

Table 3 Comparison of clinical features between variant present/absent group

Clinical feature	Odds ratio	*P*-value
Sex	2.77	0.175
First seizure (Before five years old)	8.27	**0.042**
EEG findings	∞	0.175
MRI findings	0.39	0.938
Family history	1.44	0.493
Developmental regression	1.05	0.632
Intellectual disability	2.90	0.175
ASD diagnosis	1.4	0.500
Psychiatric comorbidities	0.0	**0.033**

Comparison of clinical features was done by Fisher's Exact Test (one-sided). ASD, autism spectrum disorder.

Patient 16 harboured a maternally inherited rare likely pathogenic variant (p.Gln426Ter) in the C-terminus protein domain, which is found in the second exon of *KCNA1*. The patient's mother is mildly affected.

Patient 29 harboured a *de novo* likely pathogenic missense variant (p.Arg292His) in *STXBP1*, which was also present in his monozygotic twin, patient 30. This is a novel change at an amino acid residue where pathogenic variants have been reported previously. The patients' phenotype is compatible with *STXBP1* encephalopathy.

Patient 9: We identified a rare pathogenic missense variant (p.Gly297Arg), of unknown inheritance, in *SLC6A1*. This variant has previously been reported in a 16-year-old with myoclonic adult epilepsy, and is in keeping with this patient's phenotype.[36]

Three patients (19, 3 and 27) had three different pathogenic SNVs in *KCNA2*, but patients 3 and 27 also had *de novo* variants in other genes (*KCNIP4 and UBR5*) (Fig. 1). Clinically, all 3 patients with pathogenic *KCNA2* variants had seizures, ID and ASD, but the patients with a second *de novo* variant also had movement disorders, as described below.

Patient 19 presented with seizure onset at three years of age, mild ID, and mild ASD. She passed away from sudden unexpected death in epilepsy (SUDEP) at 26 years old. She had a novel rare pathogenic variant in *KCNA2* (p.Lys445fs).

Patient 3 presented with early onset seizure, severe ID and ASD, speech delay, dystonia, and ataxia. She had a rare pathogenic variant in *KCNA2* (p.Arg297Gln), which has previously been reported. She had a second *de novo* nonsense variant (p.Gln96Ter) impacting *KCNIP4*. This was classified as a VUS: *KCNIP4* is a gene of uncertain significance and is not associated with a disorder in humans, yet.

Patient 27 presented with seizure onset at 17 days old. She had infantile spasms, and later tonic, myoclonic, atypical absence and generalized tonic clonic seizures, evolving to a typical LGS phenotype. She also presented with severe ID, ASD, developmental delay (DD), DR, hypotonia, dysmetria and dysdiadochokinesia. Today, this patient is wheelchair bound, non-verbal, not toilet trained, and is fed through a G-tube. In addition to the rare likely pathogenic variant in *KCNA2* (p.Glu183Lys), this patient also had a *de novo* missense variant (p.Pro1309Ser, VUS) in *UBR5*. This is also a gene of uncertain significance, as *UBR5* is not associated with a disorder yet.

Copy number variants

Through WGS, we identified two clinically relevant CNVs. Patient 26 had a large likely pathogenic deletion (2 145 000 bp) on chromosome 12, spanning 38 genes.

Patient 10 is a female patient with LGS, who presented with afebrile seizure onset at three years of age (tonic, atonic, generalized tonic clonic and atypical absence). EEGs have shown fast paroxysmal activity, slow generalized sharp and wave discharges, multifocal independent spike foci and slow background. The patient's seizures are drug resistant, but she has had a moderate temporary response to a ketogenic diet. Her height and weight are below the 3rd percentile, and her head circumference is below the 2nd percentile. Previous targeted *MECP2* sequencing, metabolic investigations, an epilepsy gene panel and CMA (Affymetrix CytoScan HD SNP Array) were negative. Through WGS, a small pathogenic deletion (28 273 bp) was detected at the 2q23.1 locus, impacting both the coding and non-coding regions of *MBD5* (methyl-CpG-binding domain protein 5, OMIM 611472) and *ORC4* (origin recognition complex subunit 4, OMIM 603056). Her phenotype is in keeping with the *MBD5* CNV.

TR expansions

We identified two TR expansions in two patients with LGS: a CGG repeat expansion in the 5′ untranslated region (UTR) of *DIP2B* in patient 4 (Fig. 2), and a CTG repeat expansion (of unknown inheritance) in *ATXN8OS* in patient 6 (Fig. 3). There are no ACMG guidelines to interpret TR pathogenicity yet: therefore, these two variants were not classified, but have potential clinical implications.

Patient 4: At the age of one year, he had several febrile generalized tonic clonic seizures. At 19 months, he developed infantile spasms, and later atypical absence, myoclonic, tonic, and focal onset seizures, and EEG findings in keeping with LGS. He has significant ID, is non-verbal and not toilet trained, but can ambulate and eat unassisted. He continues to present intractable seizures of multiple types. His father had previously presented with seizures (Fig. 2A). Through WGS, a 5′UTR CGG TR expansion was identified in the brain-expressed *DIP2B* (disco-interacting protein 2 homolog B) gene. This TR expansion has previously been reported in two individuals with ID and/or seizures.[33]

We confirmed the presence of an expanded allele in patient 4 using Southern blotting (Fig. 2B, Supplementary

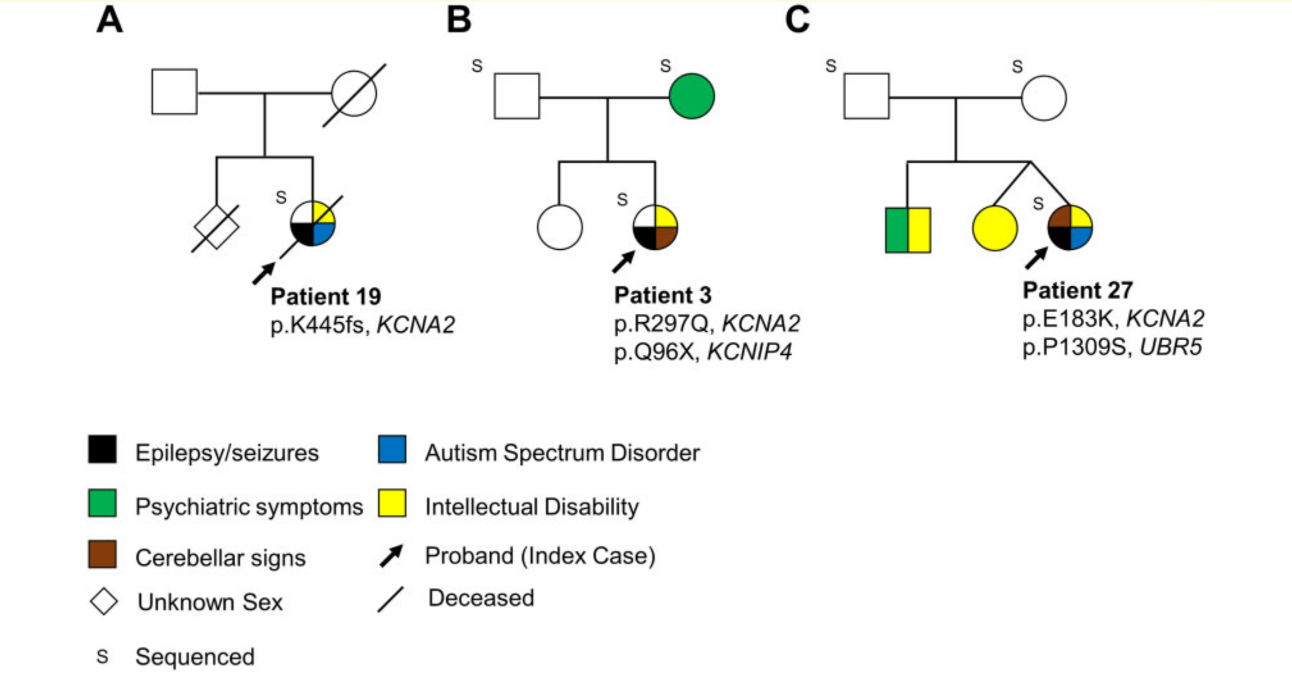

A
B
C
S
S
S
S
S
S
S
Patient 19
p.K445fs, *KCNA2*
Patient 3
p.R297Q, *KCNA2*
p.Q96X, *KCNIP4*
Patient 27
p.E183K, *KCNA2*
p.P1309S, *UBR5*
Epilepsy/seizures
Psychiatric symptoms
Cerebellar signs
Unknown Sex
Sequenced
Autism Spectrum Disorder
Intellectual Disability
Proband (Index Case)
Deceased

Figure 1 Annotated pedigrees for patients with a variant in *KCNA2*. The patient's clinical phenotype is further detailed in Table 2.

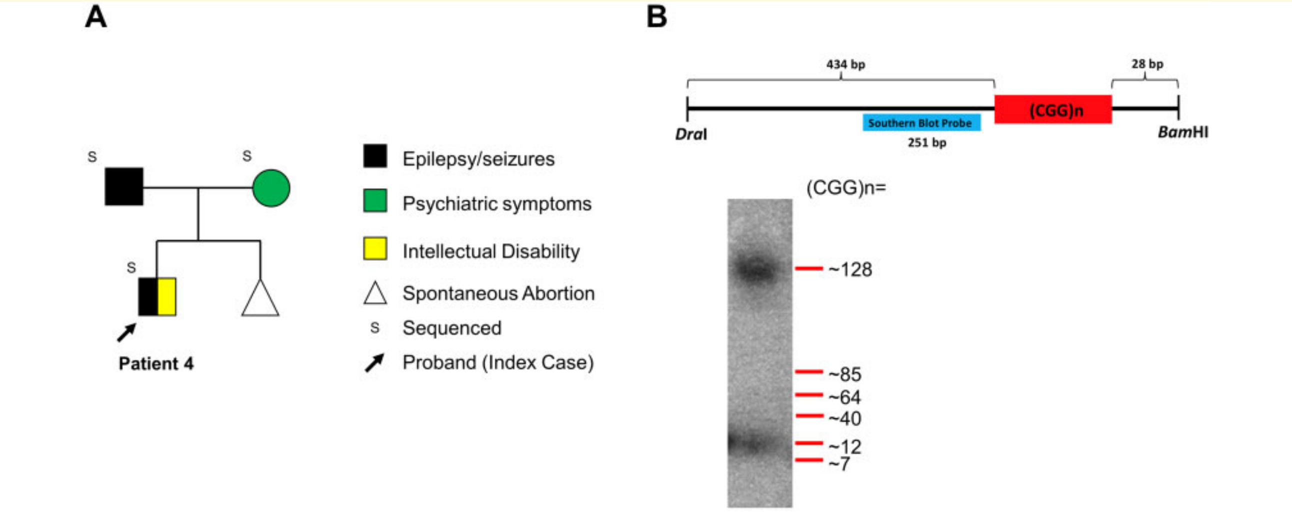

A
B
S
S
S
Patient 4
Epilepsy/seizures
Psychiatric symptoms
Intellectual Disability
Spontaneous Abortion
Sequenced
Proband (Index Case)
434 bp
28 bp
(CGG)n
DraI
BamHI
Southern Blot Probe
251 bp
(CGG)n=
~128
~85
~64
~40
~12
~7

Figure 2 Detection of a CGG expansion in *DIP2B* in patient 4. (**A**) An annotated family pedigree for patient 4 with LGS, who presented with seizures and ID. The father has a history of seizures. (**B**) Schematic shows the design, and southern blot confirmation of ∼12 and 128 CGG repeat units in the 5'UTR of *DIP2B* in patient 4.

Fig. 3). Southern blotting estimated the allele sizes to be approximately 12 and 128 CGG repeat units in patient 4, which is larger than the initial estimates by our TR detection pipeline (12 and 99 units). Our TR detection pipeline estimated 12 and 15 CGG units in the mother, and 7 and 73 CGG units in the father.

Patient 6 has a history of seizures, with initial onset at four years of age. A brain MRI scan was normal. He underwent resective surgery, guided by cortical EEG with partial resections of the left frontal, anterior parietal and left mesial temporal lobes. Pathology identified a mild disorganization of neuronal lamination with clustering of neurons. Despite this extensive resection, this patient evolved with classic LGS, exhibiting typical clinical and electrophysiological markers. Later, he also had a partial corpus callosotomy, but still has drop seizures. He does

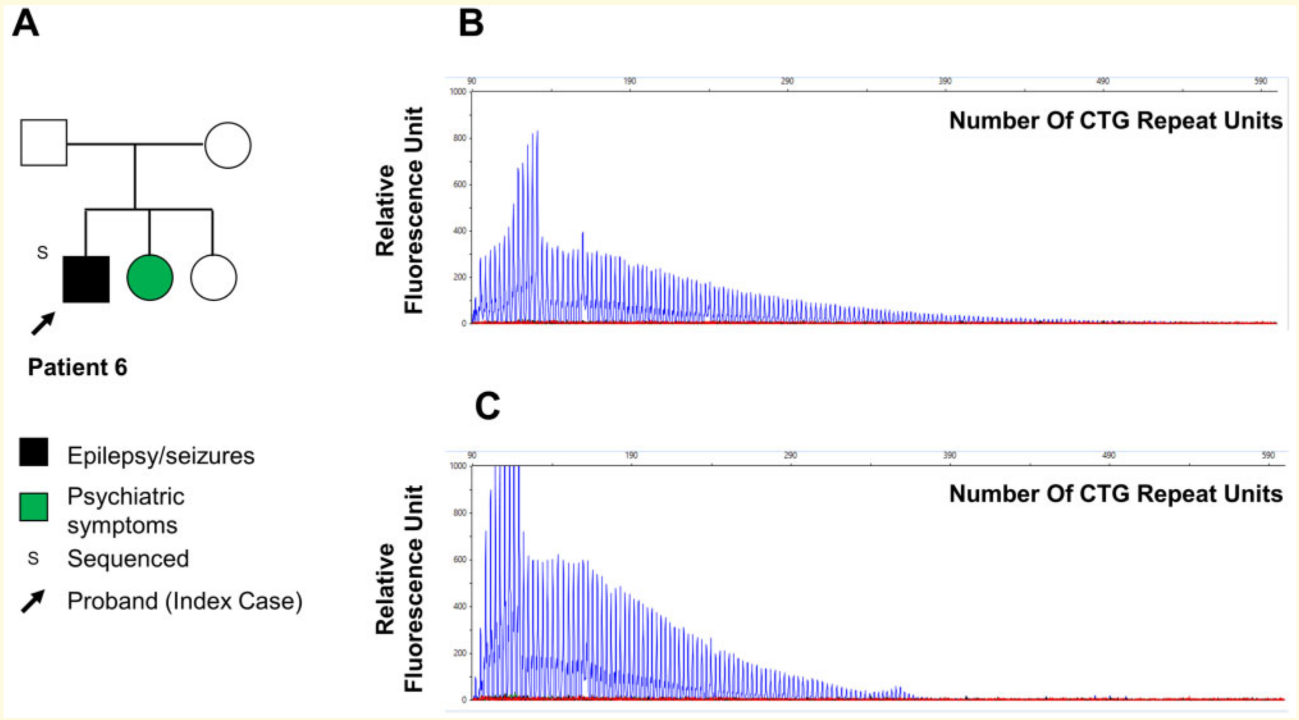


Figure 3 Detection of a CTG expansion in *ATXN80S* in patient 6. (**A**) An annotated family pedigree for patient 6, who presented with LGS. There is no family history of epilepsy. (**B**) and (**C**) display fragment length analyses of this TR locus in the patient 6 and their mother (6A), respectively, visualized using PeakScanner 2. The *x*-axis represents the fragment length (in bp) and the *y*-axis is a scale of peak intensity.

not have ataxia. Despite upper limb ataxia, his brain MRI shows a normal cerebellum, without any evidence of volume loss. Through WGS, we identified a CTG expansion in *ATXN80S*: specifically, our TR detection pipeline estimated 19 and 119 CTG repeat units in *ATXN80S*.

Using RP-PCR and capillary electrophoresis, we confirmed the presence of the expanded CTG repeats in *ATXN80S* in the proband: the repeat size is estimated to be greater than 135 CTG repeat units—the exact number cannot be determined as it is beyond the limit of this assay (Fig. 3B). We also verified the presence of a shorter expansion (~95 repeat units) in the mother (6A) (Fig. 3C). Previously, CTG TR expansions between 80 and 250 units in *ATXN80S* have been reported in spinocerebellar ataxia type 8 (SCA8).[37]

Discussion

In this study, we used WGS to evaluate a cohort of adults with unexplained DEEs. Prior to WGS, our cohort completed a total of 55 molecular genetic tests. We identified a total of nine pathogenic or likely pathogenic variants in 28 patients, resulting in a 32.1% diagnostic yield (note: this excludes VUS, such as TR expansions and SNVs impacting genes of uncertain significance). Our study demonstrates that the examination of multiple types of rare genetic variation using WGS, including those found in the non-coding region of the genome, can help resolve unexplained epilepsies.

There are variable diagnostic rates seen across different genetic testing technologies. In particular, WGS provides a more uniform distribution of coverage depth, GQ, and minor read ratio, compared to WES.[38–40] This is evident in diagnostic yields across various neurodevelopmental and neurological disorders—for example, the WES diagnostic rate in children with ASD is currently ~8.4%, but with the application of WGS, the diagnostic rate rises up to 11–25%.[41–44] WGS also provides a complete coverage of the genome; in contrast, genes selected for analysis in commercial next-generation sequencing tests can vary significantly, potentially leading to the omission of causal genetic variants.[38–40] Notably, a 100% WGS diagnostic yield has been reported in some small cohorts with specific epilepsy syndromes who previously had negative genetic tests, including a cohort of six individuals with severe-onset epilepsy, and 14 individuals with early infantile epileptic encephalopathies.[45,46] In comparison, our interrogation of multiple types of genetic variants led to a diagnostic yield of 32.1%, but could have been different had we recruited from a more homogenous cohort of adults with unexplained DEEs. For example, a genetic cause has been identified in more than 90% of patients with Dravet syndrome, while only 65 to 75% of LGS cases have a clear identifiable cause (which can include structural, metabolic or genetic causes).[13,14,47] All patients with LGS in this cohort ($n = 6$) had intractable epilepsy, including tonic seizures amongst other types, ID, and EEGs showing generalized slow spike and waves and generalized paroxysmal fast activity during sleep. WGS identified clinically relevant genetic variants in four of the six patients with LGS (66.6%). One LGS patient had a pathogenic variant in *KCNA2*, while a second had a pathogenic small CNV disrupting *MBD5 and ORC4*, which was not detected by an earlier CMA. This small deletion in the coding and non-coding regions of *MBD5* would not have been identified with current CMA technology. Two LGS patients had two independent TR expansions with clinical implications (*DIP2B and ATXN8OS*)—this is the first ever such report of TR expansions in LGS.

Novel TR expansions found in patients with LGS

For patient 6, both a CMA and gene panel did not find a genetic cause for his LGS diagnosis. Through WGS, a trinucleotide CTG repeat expansion in *ATXN8OS* in the pathogenic range was identified. *ATXN8OS* repeat expansions are associated with SCA8: a progressive ataxia exhibiting reduced penetrance, where seizures are rare, but have been reported.[37]

Upon identifying the *ATXN8OS* repeat expansion, this patient was evaluated in the ataxia clinic to determine if the falls were due to ataxia. Despite upper limb ataxia, he does not have gait ataxia nor other hallmarks of SCA8. It is unclear if the dearth of SCA8 symptoms in patient 6 is caused by low penetrance, or if he will eventually develop other symptoms of SCA8 as he ages. However, it is also possible that repeat expansions in *ATXN8OS* may manifest as LGS. This would not be completely unexpected, as both SCA8 and some other forms of SCAs also manifest with seizures and ID. For example, Swaminathan[48] reported a 22-year-old female patient with typical SCA8 who developed recurrent seizures and episodes of status epilepticus, which was successfully treated using antiepileptic therapies. In general, seizures and epilepsy are uncommon features in patients with SCAs: epilepsy as an inherent part of the phenotype has only been consistently described in SCA10 and SCA17, although an anecdotal association has also been reported in SCA2, SCA8, SCA13, SCA19/22 and SCA48.[48–52]

We found that a second LGS patient, patient 4, harboured 12 and 128 CGG repeat units in *DIP2B*, compared to 6–23 units found in controls.[33,53] This expansion has previously been reported to be associated with the fragile site FRA12A in two individuals with ID and/or seizures.[33,53] The expansion in patient 4 exceeds the length of a normal allele by over 350 base pairs (>140 excess repeat units). Interestingly, *DIP2B* is highly expressed in the brain, mainly in the frontal and parietal cortices, making it a candidate gene for an epileptic disorder.[33] The discovery of this CGG repeat expansion at 5′UTR of *DIP2B* in patient 4 adds to the limited cases in the literature, collectively suggesting that this expansion may contribute to ID and seizure-related phenotypes.[33]

To date, TR expansions have been reported as a disease-causing mechanism in over 40 disorders, including spinocerebellar ataxias.[53] To the best of our knowledge, repeat expansions have not previously been reported in LGS patients. TR expansions have been reported in patients with BAFMEs, which is associated with intronic $(TTTTA)_n(TTTCA)_n$ repeat expansions in *SAMD12, YEATS2, STARD7* and *MARCH6*.[16–19] We did not identify any such expansions in our cohort, suggesting that this may be a rare occurrence in patients with epilepsy, or is specific to BAFMEs. Interestingly, we investigated the genome-wide characteristics of TRs in 17 231 genomes of individuals with ASD, their families and control individuals, finding that TR expansions contribute a total of 2.6% risk to ASD.[26] This suggests that TR expansions are not only responsible for movement disorders, and may be a more common mechanism in neurological disorders than currently reported.

Small CNV in a patient with LGS

We also identified a CNV that was too small (28 273 bp) to be detected by a CMA in patient 10 with LGS. Individuals with damaging genetic variants impacting *MBD5* are collectively referred to as *MBD5*-associated neurodevelopmental disorders (MANDs). They exhibit DD and/or regression, ID, seizures, ASD-like behaviours, speech impairment, motor delays and hypotonia, which corresponds to patient 10's phenotype.[54] In addition, *ORC4* is implicated in the autosomal recessive Meier-Gorlin syndrome, involving short stature and microcephaly.[54] While patient 10 does not harbour a second variant impacting *ORC4*, her height and head circumference are lower than expected, suggesting that the *ORC4* disruption may be modifying the patient's overall core phenotype (which is due to *MBD5* haploinsufficiency).

Pathogenic variation in *KCNA2* associated with abnormal cerebellar findings

We identified three patients with pathogenic or likely pathogenic variants in *KCNA2*. *KCNA2* encodes a voltage-gated delayed rectifier potassium channel, and is associated with various DEEs.[55,56] Using either targeted gene sequencing or WES, Masnada et al.[56] identified variants in *KCNA2* in a cohort of 23 patients with epileptic encephalopathies, and found abnormal cerebellar findings in patients with *KCNA2* with gain-of-function (GoF) pathogenic variants.

In our cohort, out of the three carriers of variants in *KCNA2*, two had significant cerebellar signs (Patients 27 and 3). Patient 27 also harboured a VUS in *UBR5* (a ubiquitin-protein ligase component), which has previously been reported as potentially related to epilepsy, ASD and ID.[6,57–59] Previously, a missense p. Arg1907His variant was reported in a family with BAFME, which also features cerebellar dysfunction.[57] Since not all patients in the Masnada et al., 2017 cohort had undergone WES, it may be possible that some may have a variant impacting a second gene affecting cerebellar function, including those with GoF pathogenic variants in *KCNA2*. An alternative explanation is that the pathogenic variant in *KCNA2* in our patient may be the only one responsible for patient 27's phenotype, and that *UBR5* remains a gene of uncertain significance.

Patient 3 had seizures, ID, dystonia and ataxia. In addition to the GoF p. R297Q variant in *KCNA2* (which might explain the ataxia and dystonia), she also had a *de novo* VUS in *KCNIP4*.[29,55,56] *KCNIP4* encodes a potassium channel-interacting protein expressed in all regions of the brain, and is involved in regulating the frequency of slow repetitive firing and back-propagation of action potentials.[60,61] It has been suggested as a candidate gene for ADHD, epilepsy and personality disorders, though there is only a single case study and one genome-wide association study to support this.[60,61] Jenkins et al.[62] recently reported that the canine *KCNIP4* is implicated in two Norwegian Buhund dogs with progressive cerebellar ataxia, suggesting that it may be a potential candidate for cerebellar ataxia in humans and other species. Taken altogether, we propose that these additional *de novo* variants in *KCNIP4 and UBR5* genes may contribute to the more severe ID and movement disorders seen only in patients 3 and 27, but not on the other *KCNA2* variant carrier, patient 19 (who presented with epilepsy, mild ID, mild ASD and passed away from SUDEP). Sequencing *KCNIP4* and *UBR5* in larger, well-characterized cohorts will be necessary to determine their association with epilepsy, ID, and movement disorders.

Limitations

While we achieved a 32.1% diagnostic yield, it is important to note that our patients were previously screened by various next generation sequencing tests, and the overall yield would likely have been higher if there was an unbiased patient recruitment. It is also important to note that the nine rare genetic variants we identified are either disease-causing (pathogenic) or have a high likelihood to be disease-causing (likely pathogenic) for epilepsy, but may not be the sole variants contributing to the patient's phenotype (i.e. there may be other variants contributing to the patient's phenotype either independently or collectively). We did not evaluate variants with lower effect sizes (e.g. common variants) as this is a small cohort. We also did not analyse somatic variation present in resected tissues. This approach has previously identified a 'two-hit' phenomenon, where a combination of a germline and a second somatic variant can lead to focal epilepsies.[8] Finally, the two reported TR expansions cannot be classified as there are no guidelines to facilitate pathogenicity interpretation, although these expansions are in the disease range size for other diseases.[53]

Conclusion

WGS increased the diagnostic yield in our patients, previously studied with next-generation sequencing technology, by 32.1%. A majority of the identified variants (excluding those found in non-coding regions, and TR expansions) should have been detected by early next-generation sequencing tests, but were likely missed due to variability in commercial genetic testing over the years. This points to the utility of WGS in helping to resolve previously unexplained epilepsies. This is also the first report of TR expansions identified in patients with LGS, which will benefit from further functional characterization to establish causality.

Supplementary material

Supplementary material is available at *Brain Communications* online.

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Competing interests

The authors report no competing interests.

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